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RESEARCH ARTICLE

Contribution of ultrarare variants in mTOR pathway genes to sporadic focal epilepsies

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Abstract

Objective: We investigated the contribution to sporadic focal epilepsies (FE) of ultrarare variants in genes coding for the components of complexes regulating mechanistic Target Of Rapamycin (mTOR)complex 1 (mTORC1). **Methods**: We collected genetic data of 121 Italian isolated FE cases and 512 controls by Whole Exome Sequencing (WES) and single-molecule Molecular Inversion Probes (smMIPs) targeting 10 genes of the GATOR1, GATOR2, and TSC complexes. We collapsed "qualifying" variants (ultrarare and predicted to be deleterious or loss of function) across the examined genes and sought to identify their enrichment in cases compared to controls. **Results**: We found eight qualifying variants in cases and nine in controls, demonstrating enrichment in FE patients ($P = 0.006$; exact unconditional test, one-tailed). Pathogenic variants were identified in *DEPDC5* and *TSC2*, both major genes for Mendelian FE syndromes. **Interpretation**: Our findings support the contribution of ultrarare variants in genes in the mTOR pathway complexes GATOR and TSC to the risk of sporadic FE and a shared genetic basis between rare and common epilepsies. The identification of a monogenic etiology in isolated cases, most typically encountered in clinical practice, may offer to a broader community of patients the perspective of precision therapies directed by the underlying genetic cause.

Introduction

Epilepsy is a common complex disease with a clear genetic influence.[1] In the last years, next generation sequencing has proven to be a powerful tool to identify genes underlying rare Mendelian, well-recognized epilepsy syndromes at an increasingly accelerated pace.[2] Conversely, the genetic contribution to common epilepsies remains still largely unexplored and has been generally thought oligogenic or polygenic in nature. Recently, established Mendelian epilepsy genes have been

demonstrated to be major contributors to common epilepsies.[3] The risk associated with these genes is mainly attributable to ultrarare variants (e.g., unseen in the large population variant databases 1000 genomes, Exome Variant Server [EVS] and Exome Aggregate Consortium [ExAC]), which is the same class of variants primarily implicated in the Mendelian forms. Ultrarare variants of *DEPDC5* (DEP domain containing 5) are responsible for autosomal dominant focal epilepsies[4] and recently were proposed as risk factors for complex epilepsies.[3] Focal Epilepsies (FE), characterized by seizures originating within one cerebral hemisphere,[5] are the most common epilepsy form accounting for 60%–70% of all the epilepsies.[6] Familial aggregates typically have no clear-cut pattern of inheritance and, despite reflecting a more frequent clinical scenario compared to the rare multigenerational epilepsy syndromes, have long been considered poorly informative for genetic analysis.[7] In this genetic *continuum* where ultrarare variants span Mendelian and complex inheritance patterns, sporadic forms of the disease can be in principle ascribed to variation in established Mendelian genes. *DEPDC5* encodes a component of GATOR1 (Gap Activity TOward Rags 1), a protein complex that like tuberous sclerosis complex (TSC) controls the activity of mTOR complex 1 (mTORC1). Interestingly, the two *DEPDC5* GATOR1 partner genes nitrogen permease regulator-like 2 (*NPRL2*) and nitrogen permease regulator-like 3 (*NPRL3*) have been recently identified as novel familial FE genes.[8] Mutations in the two TSC complex subunit genes *TSC1/TSC2* are traditionally linked to tuberous sclerosis, an autosomal dominant multisystemic disease characterized by cortical tubers in the brain and commonly associated with FE.[9] It has been demonstrated that FE-associated mutations in this group of genes cause hyperactivation of mTORC1 signaling, suggesting a common pathological mechanism with important implications for both patients' treatment and prognosis.[4] However, if considerable knowledge has been accumulated in the last years about centrality of the mTOR pathway in familial FE, it is far less clear to what extent its deregulation impacts isolated patients. Here, we explored the contribution to sporadic, non-lesional FE of ultrarare variation in genes encoding proteins of the complexes TSC, GATOR1, and GATOR2 (the GATOR1 regulator complex) (Fig. 1). We also assayed the deregulation of mTOR pathway in patients carrying pathogenic variants in GATOR1 genes, to evaluate whether the plausible molecular mechanism underlying disease was visible in patients' lymphocytes.

Methods

Patient enrollment, collection of control samples, and study design

Following the approval by the Human Research Ethics Committee of Bellaria Hospital, Bologna (Prot. N 945/CE; cod CE: 13084), we enrolled between January 2014 and June 2017 121 Italian sporadic cases (M/F:61/60) diagnosed with non-acquired FE, according to the following criteria:

- negative family history of epilepsy within the third degree of kinship;
- no evidence of an epileptogenic lesion on brain MRI, except for hippocampal sclerosis (HS).

Most patients were diagnosed and followed up in the Bellaria Hospital of Bologna, while seven cases were referred by other Italian epilepsy centers through the Italian league against epilepsy (LICE).

Written informed consent was collected at the time of recruitment at each clinical site from patients and their healthy parents, when available.

Diagnosis of FE was based on anatomo-electro-clinical data collected during clinical assessment and made by agreement among three experts (PT, FB, and LL). Although prior genetic testing was not required for inclusion in this study, most patients have had clinically indicated genes examined in a diagnostic setting. An *ad hoc* database was created to collect patients' data, including age at seizure onset, epilepsy phenotype, personal history of febrile seizures, intellectual deficits and psychiatric disorders, focal to bilateral tonic–clonic seizures and status epilepticus, seizures frequency at last assessment, antiepileptic treatment and response to therapy, interictal epileptiform abnormalities, and neuroimaging data. All patients underwent targeted neuroradiological study with 1.5 or 3-T brain MRI acquisitions, following a specific epilepsy protocol.

DNA samples of 431 control subjects (M/F:221/210) were obtained from the Inter-Department Anti-Smoking Centre, Modena University Hospital, following the approval by the Human Research Ethics Committee of Modena (Prot. N 5554, 03/11/2013). Additionally, in silico data were collected from WES of 81 subjects (M/F:40/41) with no manifest neurological conditions enrolled in a sequencing study on inherited thrombocytopenia approved by the Ethics Committee of IRCCS San Matteo Hospital, Pavia (Prot. N 27313-2013).

Sequencing of GATOR/TSC complex genes and statistical methods

DNA was extracted from peripheral blood of patients and available parents using the QIAamp DNA Blood Mini Kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol. Genes of GATOR1 (*DEPDC5, NPRL2, NPRL3*), GATOR2 (*MIOS, SEC13, SEH1L, WDR24, WDR59*), and TSC (*TSC1, TSC2*) complexes were analyzed. *TBC1D7*, the third gene of the TSC complex, was not included in the gene set since it is linked to an ultrarare autosomal recessive macrocephaly/megalencephaly syndrome

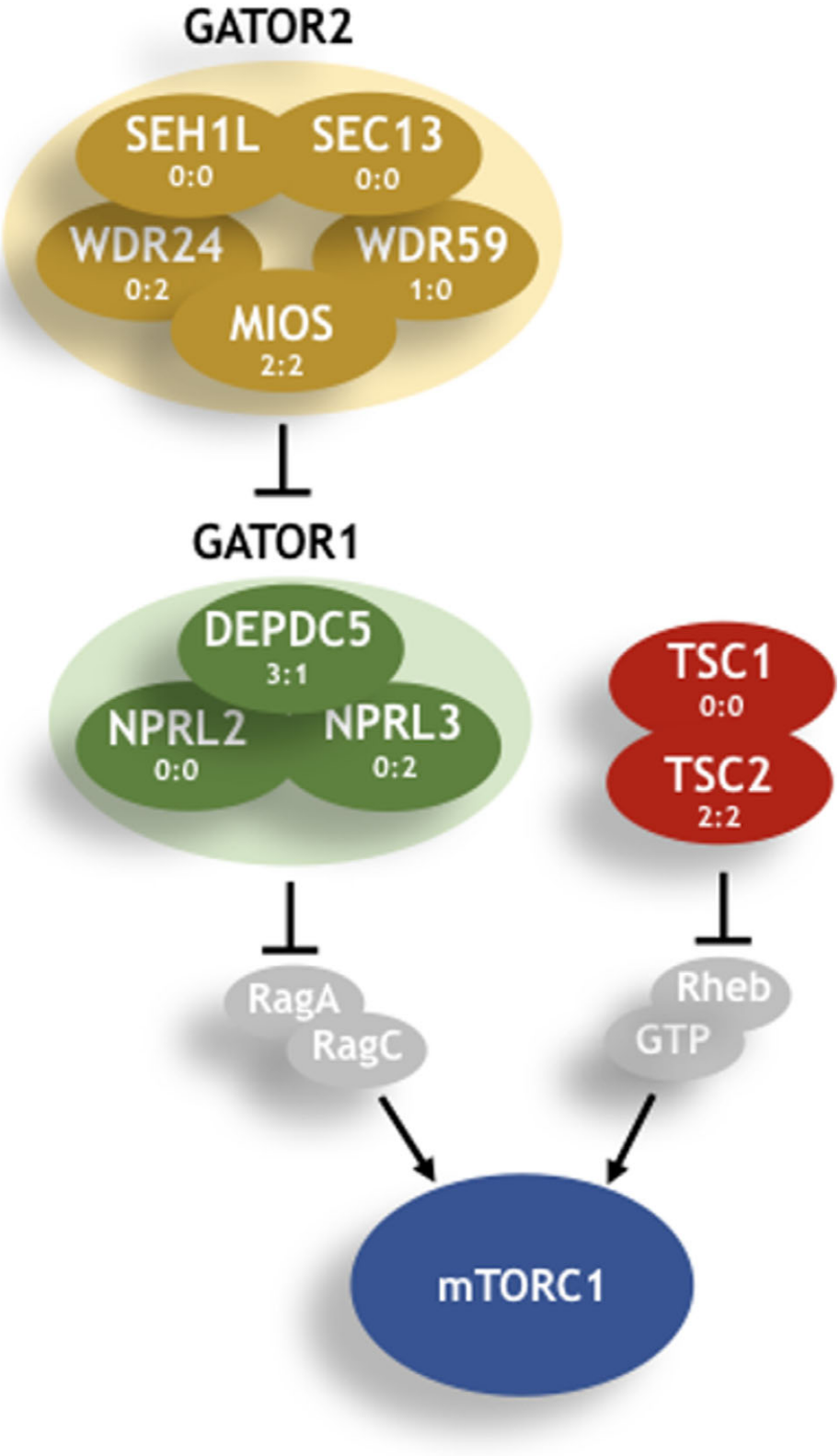


Figure 1. Simplified schematic of GATOR and TSC complexes in the mTOR pathway. The number of qualifying variants identified in each analyzed gene is reported. Under each gene name, the number of qualifying variants in cases (left) and controls (right), separated by colon.

without epilepsy.[10,11] A customized single-molecule Molecular Inversion Probes (smMIPs) assay[12,13] (Data S1) was designed using MIPgen[14] and performed following protocols already described[15] on DNA of 508 subjects, 77 FE patients and 431 Italian controls. Data for the same gene set were retrieved in silico from 125 Whole Exome Sequencing (WES) data from 44 FE patients and 81 subjects without manifest neurological conditions who were included as additional controls. Raw reads were processed as described elsewhere.[16] Variants (Single Nucleotide Variants [SNVs] and small insertions/deletions) were called using "UnifiedGenotyper" or "HaplotypeCaller"[17] from smMIPs and WES data, respectively, and variant calls were refined (Data S1) before being annotated with the Ensembl tool Variant Effect Predictor (VEP) v.76[18] against GENCODE[19] canonical transcripts on reference genome GRCh37 and additional annotations were obtained by custom scripts. We collected sequencing coverage statistics across target regions (sequence positions within coding exons and canonical splice sites of the 10 genes) with GATK "Depth of Coverage." Principal Component Analysis (PCA) was performed with EIGENSTRAT[20] to check for population structure in cases and controls (Data S1).

We defined qualifying variants, to be used for collapsing analysis comparing gene frequencies between FE patients and controls, as SNVs and small indels not observed in the ExAC, Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/), and 1000 genomes databases and for SNVs only as having a scaled C-score >=15 using Combined Annotation Dependent Depletion (CADD) v1.3.[21] We then assigned a binary variable to each subject based on absence/presence, respectively, of any number of qualifying variants per subject in the gene set. We thus modeled the number of subjects with at least one variant as a binomial distribution to identify enrichment of variants in patients compared to controls. We performed an exact unconditional test[22] based on the score statistic to test the null hypothesis of equality of proportions of subjects with at least one variant. Data analyses were performed in R3.4.2 using Package 'exact2x2' (https://www.R-project.org/). Based on ExAC data, we calculated the probability of finding a qualifying variant in any of the 10 genes among controls to be 0.01. If the true probability among cases ranges between 0.05 and 0.1, we estimated that the power to identify enrichment in the gene set with Type I error probability = 0.05 ranges between 0.65 and 0.97.

We classified as pathogenic any qualifying variant in one of the FE-associated GATOR/TSC genes (*DEPDC5, NPRL2, NPRL3, TSC1, TSC2*) if at least one of the following criteria was met:

- its predicted impact is loss of function (LOF). As GATOR1/TSC are negative regulators of mTORC1, the molecular mechanism underlying epilepsy is thought to be haploinsufficiency leading to constitutive mTORC1 activation[23];
- it had been convincingly associated with FE and/or epilepsy-related mTORopathies (focal cortical dysplasia [FCD], TSC) before.

To understand whether variants previously reported in FE, irrespective of their frequency in ExAC, were present in our cohort and could contribute to FE, we searched through the relevant literature for any variant identified among cases with ExAC MAF<=0.001.

mTOR pathway activation assay

We assessed the phosphorylation levels of the ribosomal protein S6 Kinase (S6K) on lymphocytes isolated from peripheral whole blood samples of patients with variants identified in a diagnostic setting (patients enrolled in the present genetic study were not available) and healthy controls (Data S1).

Results

Patient population

All patients had normal brain MRI except five (4.13%) with HS. Mean age at epilepsy onset (data available for 119 patients) was 16.50 ± 10.33 years (range 3 months to 55 years), mean age at last assessment 36.57 ± 13.63 years, and mean follow-up time 20.39 ± 12.24 years. Patients' features are summarized in Table 1.

Data quality checks

We removed 24 samples; all were controls sequenced by smMIPs which failed to reach required thresholds for overall on-target sequencing coverage, thus retaining 121 cases and 488 controls for downstream analysis (Fig. 2).

To exclude spurious differences in the numbers of variants between the two groups due to sequencing coverage imbalances, we verified that the mean percentage of on-target positions for each gene exceeded the required thresholds in both cases and controls (Table S1). We then compared the number of rare polymorphic SNVs and small indels (MAF <= 0.001 in population databases) between samples sequenced by WES and smMIPs, to check if merging experimental designs could affect variant comparisons between cases and controls in this study. We counted 13 variants in 125 WES (10.4%) and 56 in 484 smMIPs (11.6%) samples, resulting in no apparent discrepancy between experimental designs ($P = 0.71$,

Table 1. Clinical features of the 121 patients included in the study.

	# Patients	Valid %	Missing (%)
Gender			
Male	61	50.41	None
Female	60	49.59	
Mean age at onset	16.50 ± 10.33 years		2
Brain MRI			
Negative	116	95.87	None
HS	5	4.13	
Epilepsy type			
Temporal	64	52.89	None
Lateral	56	46.28	
Mesial	6	4.95	
Other	2	1.66	
Extra-temporal (Frontal/insular)	57	47.11	
Seizure type			
Focal, nonmotor onset	64	52.89	None
Sensory			
auditory	49	40.49	
cephalic aura	2	1.66	
Autonomic			
Gastric (rising) sensation	4	3.30	
Cognitive			
aphasic	7	5.78	
déjà vu/jamais vu	2	1.66	
Focal, motor onset	57	47.11	
hyperkinetic	37	30.58	
tonic	20	16.53	
Focal to bilateral tonic–clonic seizures	76	64.41	3 (2.48)
Seizure frequency at last assessment			
Daily/multi-daily	16	13.45	2 (1.65)
Weekly	11	9.24	
Monthly	15	12.61	
Yearly	12	10.08	
Sporadic	8	6.71	
Absent	57	47.90	
Status epilepticus	6	5.04	2 (1.65)
Epileptiform interictal EEG	64	53.33	1 (0.83)
Drug resistance	47	39.83	3 (2.48)
Personal history			
FS	11	9.24	2 (1.65)
ID/borderline IQ	7	5.93	3 (2.48)
Psychiatric disorders	18	15.13	2 (1.65)

Valid: percentage of patients with the feature. Missing: number and percentage of patients without information for the feature. HS: hippocampal sclerosis.

chi-square test). Merging WES and smMIPs data introduced therefore no substantial technological bias.

Finally, we found by PCA that cases and controls clustered together and with TSI (Italians Tuscans) and IBS (Iberians) 1000 genomes populations, identifying no population outliers in cases as well as controls. No departure from HWE was found in SNP genotypes within smMIPs or WES cohort samples (Table S2).

Enrichment and clinical relevance of qualifying variants in FE patients

We identified a total of 17 qualifying variants, eight in patients (6.6%) and nine in controls (1.8%) (Fig. 2), obtaining evidence for significant variant enrichment in patients by gene set collapsing analysis ($P = 0.006$; odds ratio: 4.25, 95% CI: 1.56–11.56). To validate our analytical strategy, we inspected the same case–control cohorts for variants in an independent set of six genes (*MTOR, CNTNAP5, SRGAP3, PIK3CA, RELN, CNTNAP2*) to various degrees implicated in neuropsychiatric disturbances including epilepsy. Following the same quality checks and filtering strategy as for the mTOR pathway gene set, we counted nine variants in cases (7.4%) and 20 in controls (4%) (Table S3) resulting in no statistically significant enrichment for variants in cases ($P = 0.10$).

Qualifying variants were distributed across 6 of the 10 analyzed genes (Fig. 1). Among FE-associated genes, three qualifying variants in *DEPDC5* and *TSC2* are to be classified as Variants of Unknown Significance (VoUS) while two variants are pathogenic and as such referenced in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/): the *TSC2* missense substitution p.Arg905Gln (dbSNP ID: rs45517259; ClinVar ID: RCV000255572.1), already associated with clinically mild tuberous sclerosis,[24] and the splice-site change c.193+1G>A (ClinVar ID: RCV000254582.1) in *DEPDC5*, previously described in patients with Familial Focal Epilepsy with Variable Foci (FFEVF)[25,26] (Table 2). Both variants occurred in patients with video-EEG documented Sleep-related Hypermotor Epilepsy (SHE), according to the novel diagnostic criteria[27] of this epilepsy syndrome, formerly known as Nocturnal Frontal Lobe Epilepsy (NFLE).

While qualifying variants were defined as not detected in ExAC, the gnomAD database (http://gnomad.broadinstitute.org/) containing a far greater set of exomes and genomes was released while this work was ongoing. Only three of the 16 qualifying variants (two in cases and one in controls) were observed in the gnomAD database, each with five or less alleles (Table 2), confirming extreme rarity of the whole variant set across human populations worldwide.

We then manually inspected patients' data to identify variants seen in ExAC that were previously reported in FE patients and found only one case with a *DEPDC5* p.Tyr281Phe variant[26] (five alleles in ExAC). However, the same variant was present also in three of our

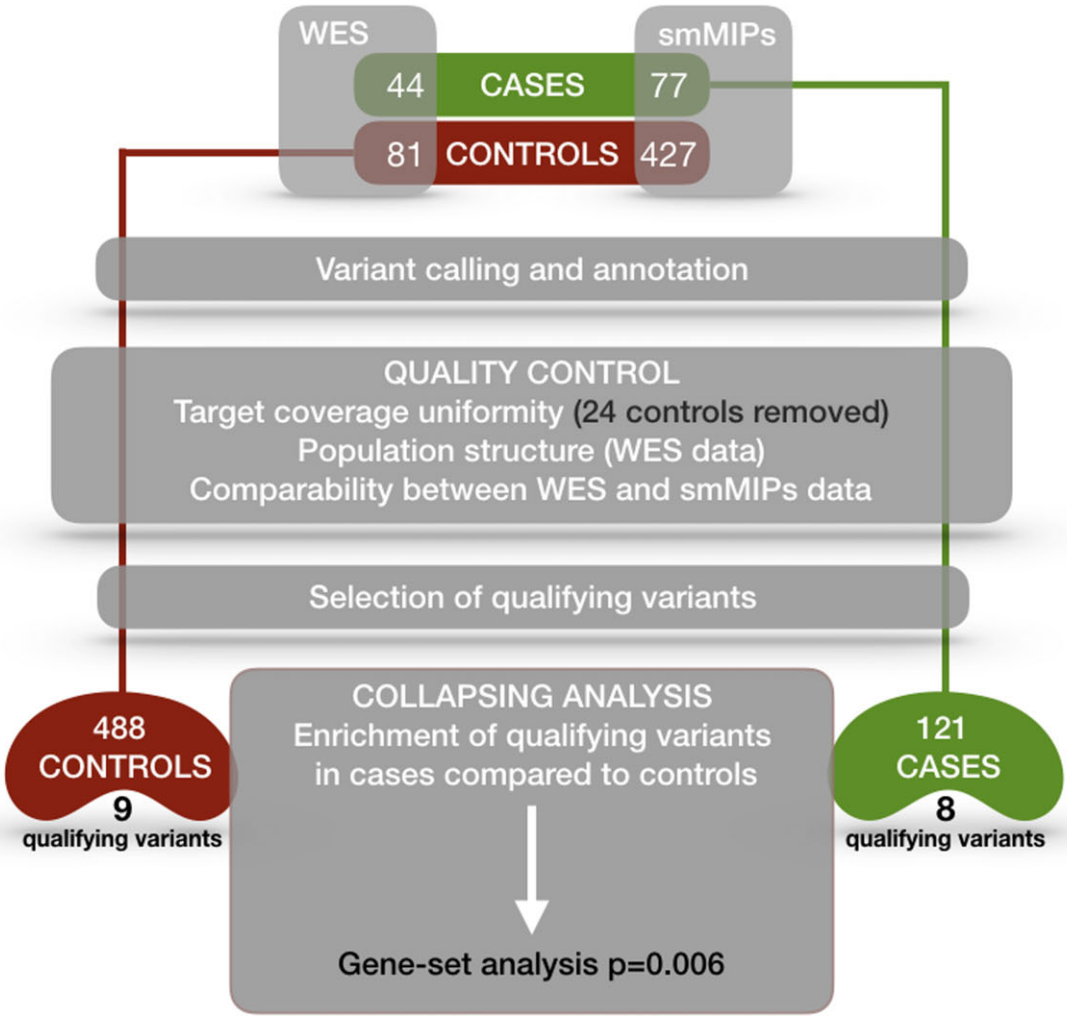


Figure 2. Workflow of the case–control gene collapsing analysis.

controls indicating that it may have MAF in the Italian population (0.003) substantially higher than in the European population as represented in ExAC/gnomAD (7x10-5).

For half patients with qualifying variants, parents' DNA was available and had been sequenced by WES allowing to evaluate the inheritance pattern. One variant (*MIOS* p.Ala138Gly) was *de novo*, while the other three (*WDR59* p.Val279Met, *DEPDC5* p.Phe1321Leu, and c.193+1G>A) were inherited. In the patient with the *de novo MIOS* event, WES revealed a pathogenic *de novo CHRNA4* p.Ser284Leu change, which had been repeatedly reported in SHE as a hot-spot mutation.[28] The *MIOS* p.Ala138Gly variant is therefore unlikely to contribute to the patient's phenotype.

Evidence of mTORC1 hyperactivation in lymphocytes of patients with GATOR1 variants

To assay mTORC1 hyperactivation in sporadic or familial FE patients, we collected fresh blood lymphocytes from available patients in whom pathogenic variants had been identified. We tested three predicted LOF changes: *DEPDC5* p.Arg165Tyr*fs**14, p.Arg422*, and *NPRL3* p.Gln101* (Fig. 3). We observed a significantly increased expression of S6K and rate of phosphorylated S6K (p-S6K) in each patient compared to controls. These findings indicate that mTORC1 hyperactivation is detectable in cells of patients with sporadic as well as familial FE. As previously reported for LOF *DEPDC5* variants,[29] we

Table 2. Qualifying variants.

Subject type	Variant	Gene	Ex	mRNA change	AA change	CADD	CR	gnomAD MAF (alleles)	Inheritance
Case	chr22-32180826-A-G	DEPDC5	9	c.A589G	p.Asn197Asp	27.1	V	-	N.A.
Case	chr22-32275693-T-C	DEPDC5	37	c.T3961C	p.Phe1321Leu	25.0	V	-	Inherited
Case	chr22-32156689-G-A	DEPDC5	3	c.193+1G>A	-	25.9	P	-	Inherited
Control	chr22-32275619-A-T	DEPDC5	37	c.A3887T	p.His1296Leu	22.8	V	-	N.A.
Case	chr7-7629178-G-A	MIOS	9	c.G2027A	p.Ser676Asn	26.3	V	-	N.A.
Case	chr7-7612519-C-G	MIOS	4	c.C413G	p.Ala138Gly	23.7	V	-	De Novo
Control	chr7-7612698-A-G	MIOS	4	c.A592G	p.Lys198Glu	24.5	V	-	N.A.
Control	chr7-7636048-C-T	MIOS	11	c.C2357T	p.Ala786Val	34	V	-	N.A.
Control	chr16-136824-C-A	NPRL3	14	c.G1587T	p.Met529Ile	31	V	-	N.A.
Control	chr16-160550-G-A	NPRL3	6	c.C602T	p.Ala201Val	28.0	V	-	N.A.
Case	chr16-2124390-A-G	TSC2	22	c.A2545G	p.Thr849Ala	23.0	V	8.237e-06 (2)	N.A.
Case	chr16-2126143-G-A	TSC2	24	c.G2714A	p.Arg905Gln	34	P	-	N.A.
Control	chr16-2114391-C-G	TSC2	15	c.C1562G	p.Thr521Arg	28.4	V	3.229e-05 (1)	N.A.
Control	chr16-2122943-G-A	TSC2	20	c.G2314A	p.Ala772Thr	23.7	V	-	N.A.
Control	chr16-735303-A-G	WDR24	7	c.T1973C	p.Ile658Thr	24.3	V	-	N.A.
Control	chr16-735306-A-G	WDR24	7	c.T1970C	p.Leu657Pro	25.6	V	-	N.A.
Case	chr16-74955896-C-T	WDR59	10	c.G835A	p.Val279Met	34	V	2.031e-05 (5)	Inherited

Variants are annotated according to Ensembl transcripts. DEPDC5:ENST00000382112; MIOS: ENST00000340080; NPRL2: ENST00000232501; NPRL3: ENST00000399953; TSC2: ENST00000219476; WDR24: ENST00000293883; WDR59: ENST00000262144. Ex: Exon C.R.: Clinical Relevance. V: VoUS; P: pathogenic. gnomAD release used is r2.0.2.

provide evidence that *NPRL3* LOF variants impact the mTOR pathway as well.

Discussion

Few reports have been published on the identification of mTOR pathway gene variants in large (>100 subjects) collections of sporadic FE patients. These studies were restricted to *DEPDC5*[30,31] or GATOR1 complex genes[8] and did not provide estimates of the risk for FE that the identified variants accounted for.

Here we describe the case–control sequencing strategy we used to address this shortcoming in the literature, and we demonstrate that genes of the GATOR/TSC complexes are collectively enriched for qualifying variants in sporadic FE patients compared to population-matched controls. In 6.6% of cases versus 1.8% of controls, we identified qualifying variants conferring a higher than fourfold risk of FE.

Even taking into account only those variants considered as pathogenic according to our stringent criteria, their percentage in cases (1.7%) is in line with that ascribed to genes associated with distinctive FE phenotypes in the respective sporadic forms (*LGI1* in epilepsy with auditory features [EAF][32] or nicotinic acetylcholine receptors [*CHRNA4, CHRNA2, CHRNB2*] in SHE).[33] This suggests that the risk ascribable to GATOR/TSC genes is at least as large as that of major established FE genes.

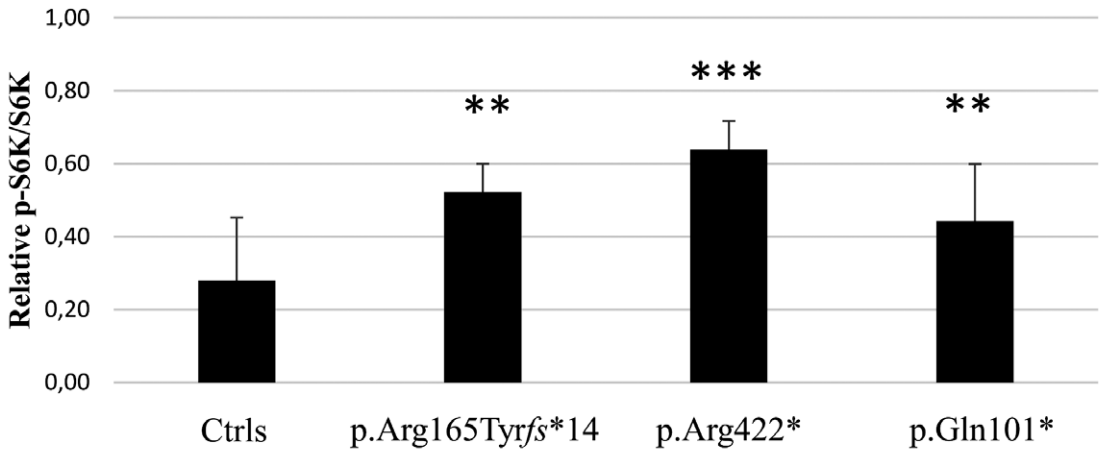
The pathogenic variants we identified had been before convincingly associated with FE or related mTORopathies. The splice-site variant in *DEPDC5* has a predicted LOF impact and therefore also fits with the recognized molecular mechanism of pathogenicity for this gene, which is haploinsufficiency thought to lead to mTORC1 hyperactivation. This variant resulted to be inherited from a healthy parent. Similarly, the few sporadic FE patients previously reported had often inherited the causative variant,[8,30,31] which is congruent with the largely documented incomplete penetrance in families.[8,25,34,35] Unlike severe, early-onset epilepsies such as epileptic encephalopathies,[36] the pathogenic potential of a variant should not be undervalued in FE based on the fact that it did not occur *de novo*. It is rather more relevant that the variant is ultrarare, as it has been observed that most variants involved in epilepsy have been seen ever or only once in ExAC.[37]

Interestingly, both pathogenic variants were identified in patients with SHE. The patient with *TSC2* p.Arg905Gln had refractory SHE without evidence of TSC-related white matter lesions on brain MRI. p.Arg905 is a mutational hot spot and the same change has been reported in mild familial and sporadic TSC presenting with hypomelanotic macules or focal seizures with spontaneous remission or easily controlled with medication.[24,38] We reviewed the epilepsy phenotypes previously associated with p.Arg905Gln among which focal seizures with possible generalization was the most frequent seizure type, while only few patients had prevalent sleep-related events. Thus, we enlarged the *TSC2* p.Arg905Gln mutational spectrum to non-lesional SHE.

A

Gene	Variant	Inheritance	Individuals analyzed
DEPDC5	p.Arg165Tyr fs*14	Sporadic	1
DEPDC5	p.Arg422*	Familial	3
NPRL3	p.Gln101*	Familial	2

B



C

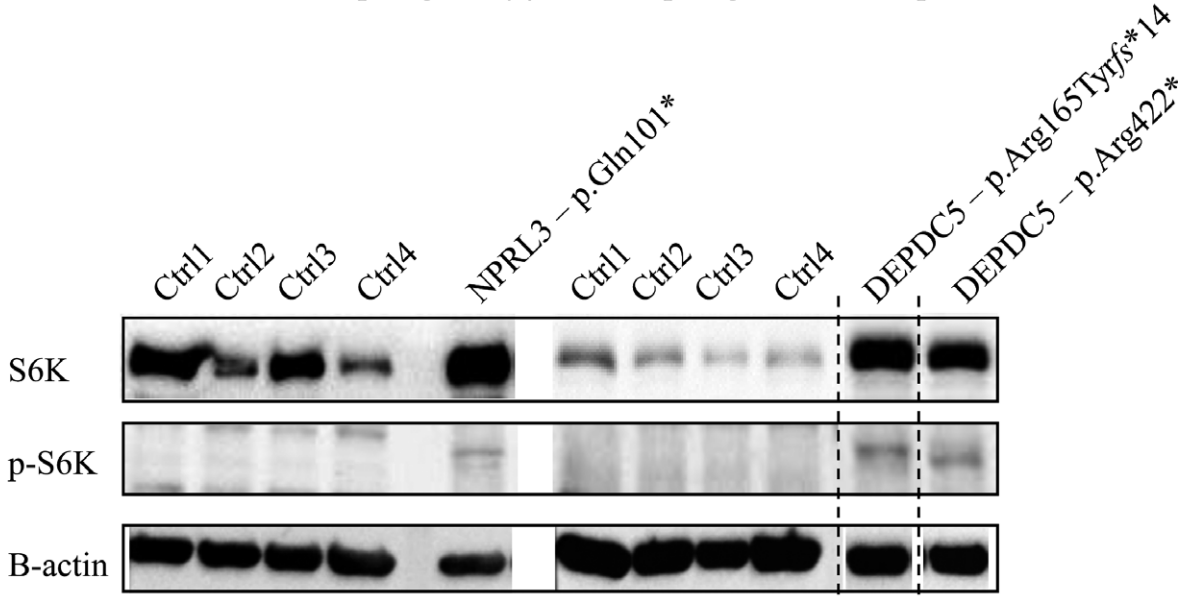


Figure 3. Functional assessment of mTORC1 activation in human lymphocytes. (A) List of the evaluated variants; (B) densitometric analysis of western blot showing the mean ratio of p-S6K over total S6K expression in the tested lymphocytes; (C) representative western blot result. In panel (B), asterisks indicate significant *P*-values obtained when comparing patients and controls (two-tailed T-test): ***P*-value < 0.01, ****P*-value < 0.001.

DEPDC5 c.193+1G>A had been identified in affected members of a FFEVF pedigree showing different patterns of FE,[25,26] including frontal lobe epilepsy, occipital lobe epilepsy with previous infantile spasms (IS), and severe multifocal epilepsy with IS, ID, and autistic features. None of the affected members had SHE.

The occurrence of both pathogenic variants in patients with SHE raises the question whether GATOR/TSC

complex genes may be more likely implicated in specific FE phenotypes. A relatively higher risk for SHE in particular is an intriguing hypothesis because of the well-known association between this group of genes and epilepsy related to focal cortical dysplasias (FCD), predominantly manifesting with sleep-related seizures which arise within the frontal lobe.[39] *DEPDC5* and *TSC2* mutations have been already reported in sporadic and familial FCD.[8,26,40–45] Although the present study was focused on non-lesional FE, it is still possible that a subtle FCD could not be detected by conventional brain MRI in the two patients with pathogenic variants.

Most variants for which GATOR/TSC genes are enriched in our cases are missense. This suggests that, together with LOF variants, this class of variation contributes to the risk of FE. However, ultrarare or private missense variants still pose an important problem for risk assessment, especially in isolated patients where segregation data cannot be supportive. Except for *TSC2* p.Arg905Gln, all missense variants were indeed classified as VoUS.

Although adaptation of the ACMG criteria to the specific situation of GATOR1 variants has proven useful to increase the accuracy of the clinical classification,[46] interpretation of missense variants remains a major challenge.

Our functional data show that mTOR pathway deregulation, as measurable in our *ex vivo* assay, was similar in cells of familial and sporadic FE patients carrying a LOF variant. We also provided evidence that, as for DEPDC5, also NPRL3-predicted haploinsufficiency was associated with the plausible molecular mechanism of disease. Functional assessments may be therefore of great help in reclassifying missense variants in GATOR/TSC genes based on their effect on mTORC1 activity.[29,47] Correct classification of the variant pathogenic potential is very important for the clinical use. Drugs targeting the mTOR pathway, candidate for treating patients with rare familial mTORopathies, may be effective also in the more common sporadic FE where the underlying defect is in any of different genes converging on deregulation of the mTOR signaling pathway.

Although we retained only variants ever seen in ExAC in the case–control study, we wanted to understand whether variants previously reported in FE patients, irrespective of their frequency in ExAC, were present also in our cohort and could contribute to the risk of FE. We identified only one case with such a variant in *DEPDC5* that had been previously reported to segregate in a FE family.[26] The identification of the same variant also in three of our controls suggests that it has higher frequency in the Italian population than in ExAC/gnomAD, and therefore diminishes its potential implication in FE. This highlights that sequencing control cohorts from local populations (such as the Italian, not extensively represented in public variant databases) may also be useful in reclassifying variants.

From a technical perspective, smMIPs proved to be as informative as high-coverage WES, highly comparable in terms of the effectiveness of variant detection. smMIPs have also been shown to accurately detect variants with low allelic fraction in the sample.[13] Although we identified no seemingly mosaic variant in our cohorts, it is possible that sequencing by smMIPS with higher depth of coverage would help to investigate the role of post-zygotic variants appearing at low fraction in the sequenced sample.

In conclusion, our findings support the contribution of ultrarare, likely deleterious variants in genes of the mTOR pathway regulators GATOR and TSC to the risk of sporadic, non-lesional FE. Our strategy to collapse qualifying variants at the level of the gene set, not only of the single gene, allowed to reveal that a group of functionally interrelated genes were collectively enriched for variants in this cohort of sporadic FE patients. We identified pathogenic variants in major genes responsible for familial FE demonstrating a shared genetic basis between rare Mendelian syndromes and the more common sporadic forms. Most missense changes were classified as VoUS, and functional assays for the detection of mTOR pathway hyperactivation may be useful in discriminating variants with a pathogenic potential from the presumably benign ones. The identification of a monogenic etiology in isolated cases, who are the most typically encountered in the clinical practice, may offer to a broader community of patients the perspective of therapies precisely targeted to the underlying genetic cause.

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Author Contributions

TP, LL, and FB involved in conception and design of the study. TP, LL, SB, CMa, MDL, CMy, EN, FP, CC, RM, EB, LG, GC, and GdO involved in acquisition and analysis of data. TP, LL, SB, and CMa drafted the manuscript, tables, and figures. MS, GG, HCM, and PT revised the manuscript for important intellectual content.

Conflict of Interest

The authors report no disclosures.

References

1. Hildebrand MS, Dahl HH, Damiano JA, et al. Recent advances in the molecular genetics of epilepsy. J Med Genet 2013;50:271–279.
2. Hardies K, Weckhuysen S, De Jonghe P, Suls A. Lessons learned from gene identification studies in Mendelian epilepsy disorders. Eur J Hum Genet 2016;24:961–967.
3. Epi4K consortium; Epilepsy Phenome/Genome Project. Ultra-rare genetic variation in common epilepsies: a case-control sequencing study. Lancet Neurol 2017;16:135–143.
4. Baldassari S, Licchetta L, Tinuper P, et al. GATOR1 complex: the common genetic actor in focal epilepsies. J Med Genet 2016;53:503–510.
5. Berg AT, Berkovic SF, Brodie MJ, et al. Revised terminology and concepts for organization of seizures and epilepsies: report of the ILAE commission on classification and terminology, 2005-2009. Epilepsia 2010;51:676–685.
6. Panayiotopoulos CP. The Epilepsies: Seizures, Syndromes and Management. Oxfordshire (UK): Bladon Medical Publishing, 2005.
7. Pippucci T, Licchetta L, Baldassari S, et al. Epilepsy with auditory features: A heterogeneous clinico-molecular disease. Neurol Genet 2015 May 14;1:e5.
8. Ricos MG, Hodgson BL, Pippucci T, et al. Mutations in the mammalian target of rapamycin pathway regulators NPRL2 and NPRL3 cause focal epilepsy. Ann Neurol 2016;79:120–131.
9. Curatolo P, Moavero R, Roberto D, Graziola F. Genotype/phenotype correlations in tuberous sclerosis complex. Semin Pediatr Neurol 2015;22:259–273.
10. Alfaiz AA, Micale L, Mandriani B, et al. TBC1D7 mutations are associated with intellectual disability, macrocrania, patellar dislocation, and celiac disease. Hum Mutat 2014;35:447–451.
11. Capo-Chichi JM, Tcherkezian J, Hamdan FF, et al. Disruption of TBC1D7, a subunit of the TSC1-TSC2 protein complex, in intellectual disability and megalencephaly. J Med Genet 2013;50:740–744.
12. Porreca GJ, Zhang K, Li JB, et al. Multiplex amplification of large sets of human exons. Nat Methods 2007;4:931–936.
13. Hiatt JB, Pritchard CC, Salipante SJ, et al. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 2013;23:843–854.
14. Boyle EA, O'Roak BJ, Martin BK, et al. MIPgen: optimized modeling and design of molecular inversion probes for targeted resequencing. Bioinformatics 2014;30:2670–2672.
15. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619–1622.
16. Magini P, Pippucci T, Tsai IC, et al. A mutation in PAK3 with a dual molecular effect deregulates the RAS/MAPK pathway and drives an X-linked syndromic phenotype. Hum Mol Genet 2014;23:3607–3617.
17. derVan Auwera GA, Carneiro MO, Hartl C, et al. From FastQ data to high confidence variant calls: the genome analysis toolkit best practices pipeline. Curr Protoc Bioinformatics 2013;43:11.10.1–11.10.33.
18. McLaren W, Gil L, Hunt SE, et al. The ensembl variant effect predictor. Genome Biol 2016;17:122.
19. Harrow J, Frankish A, Gonzalez JM, et al. GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res 2012;22:1760–1774.
20. Price AL, Patterson NJ, Plenge RM, et al. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006;38:904–909.
21. Kircher M, Witten DM, Jain P, et al. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet 2014;46:310–315.
22. Mehrotra DV, Chan IS, Berger RL. A cautionary note on exact unconditional inference for a difference between two independent binomial proportions. Biometrics 2003;59:441–450.
23. Crino PB. mTOR signaling in epilepsy: insights from malformations of cortical development. Cold Spring Harb Perspect Med 2015;5:a022442.
24. Jansen AC, Sancak O, D'Agostino MD, et al. Unusually mild tuberous sclerosis phenotype is associated with TSC2 R905Q mutation. Ann Neurol 2006;60:528–539.
25. Dibbens LM, de Vries B, Donatello S, et al. Mutations in DEPDC5 cause familial focal epilepsy with variable foci. Nat Genet 2013;45:546–551.
26. Carvill GL, Crompton DE, Regan BM, et al. Epileptic spasms are a feature of DEPDC5 mTORopathy. Neurol Genet 2015;1:e17.
27. Tinuper P, Bisulli F, Cross JH, et al. Definition and diagnostic criteria of sleep-related hypermotor epilepsy. Neurology 2016;86:1834–1842.
28. Hirose S, Iwata H, Akiyoshi H, et al. A novel mutation of CHRNA4 responsible for autosomal dominant nocturnal frontal lobe epilepsy. Neurology 1999;53:1749–1753.
29. van Kranenburg M, Hoogeveen-Westerveld M, Nellist M. Preliminary functional assessment and classification of DEPDC5 variants associated with focal epilepsy. Hum Mutat 2015;36:200–209.
30. Lal D, Reinthaler EM, Schubert J, et al. DEPDC5 mutations in genetic focal epilepsies of childhood. Ann Neurol 2014;75:788–792.
31. Tsai MH, Chan CK, Chang YC, et al. DEPDC5 mutations in familial and sporadic focal epilepsy. Clin Genet 2017;92:397–404.
32. Nobile C, Michelucci R, Andreazza S, et al. LGI1 mutations in autosomal dominant and sporadic lateral temporal epilepsy. Hum Mutat 2009;30:530–536.

33. Marini C, Guerrini R. The role of the nicotinic acetylcholine receptors in sleep-related epilepsy. Biochem Pharmacol 2007;74:1308–1314.
34. Ishida S, Picard F, Rudolf G, et al. Mutations of DEPDC5 cause autosomal dominant focal epilepsies. Nat Genet 2013;45:552–555.
35. Picard F, Makrythanasis P, Navarro V, et al. DEPDC5 mutations in families presenting as autosomal dominant nocturnal frontal lobe epilepsy. Neurology 2014;82:2101–2106.
36. Epi4K Consortium; Epilepsy Phenome/Genome Project, Allen AS, Berkovic SF, Cossette P, et al. De novo mutations in epileptic encephalopathies. Nature 2013;501:217–221.
37. Bennett CA, Petrovski S, Oliver KL, Berkovic SF. ExACtly zero or once: A clinically helpful guide to assessing genetic variants in mild epilepsies. Neurol Genet 2017;3:e163.
38. Tyburczy ME, Jozwiak S, Malinowska IA, et al. A shower of second hit events as the cause of multifocal renal cell carcinoma in tuberous sclerosis complex. Hum Mol Genet 2015;24:1836–1842.
39. Nobili L, Cardinale F, Magliola U, et al. Taylor's focal cortical dysplasia increases the risk of sleep-related epilepsy. Epilepsia 2009;50:2599–2604.
40. Scheffer IE, Heron SE, Regan BM, et al. Mutations in mammalian target of rapamycin regulator DEPDC5 cause focal epilepsy with brain malformations. Ann Neurol 2014;75:782–787.
41. Baulac S, Ishida S, Marsan E, et al. Familial focal epilepsy with focal cortical dysplasia due to DEPDC5 mutations. Ann Neurol 2015;77:675–683.
42. D'Gama AM, Geng Y, Couto JA, et al. Mammalian target of rapamycin pathway mutations cause hemimegalencephaly and focal cortical dysplasia. Ann Neurol 2015;77:720–725.
43. Scerri T, Riseley JR, Gillies G, et al. Familial cortical dysplasia type IIA caused by a germline mutation in DEPDC5. Ann Clin Transl Neurol 2015;2:575–580.
44. Weckhuysen S, Marsan E, Lambrecq V, et al. Involvement of GATOR complex genes in familial focal epilepsies and focal cortical dysplasia. Epilepsia 2016;57:994–1003.
45. Lim JS, Gopalappa R, Kim SH, et al. Somatic mutations in TSC1 and TSC2 cause focal cortical dysplasia. Am J Hum Genet 2017;100:454–472.
46. Baldassari S, Picard F, Verbeek NE, et al. The landscape of epilepsy-related GATOR1 variants. Genet Med 2018 https://doi.org/10.1038/s41436-018-0060-2.
47. Hoogeveen-Westerveld M, Wentink M, van den Heuvel D, et al. Functional assessment of variants in the TSC1 and TSC2 genes identified in individuals with tuberous sclerosis complex. Hum Mutat 2011;32:424–435.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplementary Methods
Table S1. Depth of coverage statistics for WES and smMIPs
Table S2. Exact test of Hardy–Weinberg Equilibrium for 85 biallelic polymorphic sites within WES and smMIPs cohort samples
Table S3. Qualifying variants identified among cases and controls in an independent set of six genes)

Appendix

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